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NIGHT/DAY CHANGES IN PINEAL EXPRESSION OF >600 GENES: CENTRAL ROLE OF ADRENERGIC/cAMP SIGNALING

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The pineal gland plays an essential role in vertebrate chronobiology by converting time into a hormonal signal, melatonin, which is always elevated at night. Here we have analyzed the rodent pineal transcriptome using Affymetrix GeneChip® technology to obtain a more complete description of pineal cell biology. The effort revealed that 604 genes (1,268 probe sets) with Entrez Gene identifiers are differentially expressed greater than 2-fold between mid-night and mid-day (False discovery rate < 0.20). Expression is greater at night in ~ 70 percent. These findings were supported by the results of radiochemical *in situ* hybridization histology and qRT-PCR studies. We also found that the regulatory mechanism controlling the night/day changes in the expression of most genes involves norepinephrine-cyclic AMP signaling. Comparison of the pineal gene expression profile to that in other tissues, identified 334 genes (496 probe sets) that are expressed greater than 8-fold higher in the pineal gland relative to other tissues. Of these genes, 17% are expressed at similar levels in the retina, consistent with a common evolutionary origin of these tissues. Functional categorization of the highly expressed and/or night/day differentially expressed genes identified clusters that are markers of specialized functions including the immune/ inflammation response, melatonin synthesis, photodetection, thyroid hormone signaling and diverse aspects of cellular signaling and cell biology. These studies produce a paradigm shift in our understanding of the 24-hour dynamics of the pineal gland from one focused on melatonin synthesis to one including many cellular processes.

A defining feature of the pineal gland is a 24-hour rhythm in melatonin synthesis. Melatonin provides vertebrates with a circulating signal of time and is essential for optimal integration of physiological functions with environmental lighting on a daily and seasonal basis (1-4).

The melatonin rhythm in mammals is driven by a circadian clock located in the suprachiasmatic nucleus (SCN), which is hard-wired to the pineal gland by a polysynaptic pathway that courses through central and peripheral neuronal structures. The pineal gland is innervated by projections from the superior cervical ganglia (SCG) in the form of a dense network of catecholamine-containing sympathetic fibers. Activation of the SCN → pineal pathway occurs at night and results in the release of norepinephrine (NE) from the sympathetic fibers into the pineal perivascular space (5). NE activates the pinealocyte through adrenergic receptors (5,6). The best studied mechanism involves coincident "AND" gate activation of α_{1b}- and β_1- adrenergic receptors, which maximally stimulates adenylate cyclase, thereby elevating cAMP (7-13). Activation of α_{1b}- adrenergic receptors alone elevates intracellular calcium and phospholipid signaling (1,14-16).

cAMP is believed to mediate the effects of NE on melatonin production, to a large part by activating cAMP-dependent protein kinase (PKA). In rodents, this induces expression of *Aanat,* the penultimate enzyme in melatonin synthesis (17). Induction occurs through phosphorylation of cAMP response element binding protein (CREB) bound to cAMP response elements (CREs) in the *Aanat* gene. A similar NE/cAMP mechanism also controls expression of *Adra1b, Atp7b*, *Crem*[1], *Dio2*, *Fosl2*, *Id1*, *Dusp1*, *Mat2a*, *Nr4a1*, *Slc15a1*, *Pde4b2, Ptch1* and *Rorb* (18-27). In addition, a NE/cAMP mechanism decreases expression of *Hs3st2* (28). Although it is likely that some of the effects of cAMP involve CREs, it is also likely that cAMP influences pineal gene expression through epigenetic mechanisms that alter chromatin structure, e.g. histone phosphorylation (29,30), thereby having the potential of altering the expression of many genes and broadly promoting transcription by factors other than CREB. Whereas there is abundant evidence

that the SCN/SCG/NE/cAMP system controls rhythmic gene expression in the pineal gland, it is also possible that other regulatory mechanisms exist, involving release of other transmitters, and additional second messengers (e.g. cGMP, Ca^{++}, phospholipids).

The increased abundance of some of these night/day differentially expressed genes and of other genes in the pineal gland is determined in part by members of the OTX2/CRX family of homeodomain proteins, which play a similar role in the retina (31-34). These factors bind to photoreceptor conserved elements and closely related sequences (PCEs). In addition, *Pax6* and *Otx2* are essential for development of both tissues (35-37). This developmental similarity is consistent with the common evolutionary origin of the pineal gland and retina from a primitive photodetector (38). Examples of OTX2/CRX-controlled genes expressed in both tissues include *Aanat, Asmt, Sag,* and *Grk1* (20,39-46). The first two encode proteins dedicated to melatonin synthesis; the latter two encode proteins associated with phototransduction in the retina. It is not clear whether the proteins encoded by these phototransduction genes play parallel roles in NE/cAMP signal transduction in the pinealocyte or if they are functionally vestigial in the context of the pinealocyte. Although OTX2 and CRX are of central importance in these tissues, it appears that other transcription factors and regulatory cascades are involved. For example, the importance of E-boxes in determining tissue-specific expression of *Aanat* is evident from several studies (42,47); and NeuroD1 may also play a role in determining pineal-specific expression patterns (48).

Whereas in both the pineal gland and retina, PCEs control developmental expression of the same gene, different mechanisms can operate in each tissue to control rhythmicity. For example, in the case of *Aanat,* CREs mediate cAMP control of 24-hour rhythms in the pineal gland (49,50). In the retina, however, E-box elements mediate circadian clock control of the 24-hour rhythm in *Aanat* expression (51).

In addition to the accepted SCN/SCG/NE/cAMP pathway, reports in the literature have claimed that a circadian clock regulates daily changes in the expression of some genes in the mammalian pineal gland (52), as in the submammalian pineal gland (53,54). The physiological impact of this remains unknown.

Here we have expanded our understanding of the transcriptional regulation and physiology of the pineal gland by employing Affymetrix GeneChip® technology, including a microarray that interrogates more than 13,663 genes that have been assigned Entrez Gene identifiers[2]. Previous studies of this nature in the rat have identified 39 night/day differentially expressed genes (26); a more recent study identified 35 such genes with Entrez Gene identifiers (59 probe sets) (55). Our study had three specific goals. The first was to produce a comprehensive listing of genes that are differentially expressed on a night/day basis. The second was to identify the highly enriched genes that define pineal function, independent of whether they are tonically or night/day differentially expressed; this was done by comparing gene expression in the pineal gland to median expression among other tissues. The third goal was to determine the scope of the NE/cAMP regulatory cascade; this approach utilized an *in vitro* organ culture system. In addition to establishing the importance of this cascade, the organ culture studies identified sets of genes that were spontaneously up- or down-regulated more than 10-fold during culture in defined medium, providing evidence of the existence of unknown regulatory mechanisms. An unexpected discovery was that the pineal transcriptome includes a large number of immune/inflammation response-associated genes.

The findings of this report are of value to investigators interested in the pineal gland, chronobiology, neuroendocrinology and immunology and to those who study specific genes that are night/day differentially and/or highly expressed in the pineal gland.

EXPERIMENTAL PROCEDURES

Animals: Three microarray experiments were done (experiments A, B and C). For microarray experiments A and B (Cardiff University), Sprague-Dawley rats (2 to 3 months old) were maintained in standard laboratory conditions in a LD 14:10 light–dark cycle (lights on: 05:00 h). Animals were killed at mid-day (ZT7) or mid-night (ZT19) by cervical dislocation and pineal glands were rapidly dissected, placed in tubes on solid CO_2, and stored at -80ºC. For microarray experiment C (NICHD), for the time series analysis of gene expression by qRT-PCR (Figure 4), and for organ culture experiments, Sprague-Dawley rats (2 to 3 months old, female) were housed for two weeks in LD 14:10 lighting cycles, killed by CO_2 asphyxiation and decapitated; pineal glands were rapidly dissected, and either placed in tubes on solid CO_2, and stored at -80ºC, or prepared for organ culture. Other tissues were also removed, and 10 mg samples were frozen and stored in a similar manner. For the qRT-PCR experiment (Figure 4), tissues were collected at ZT1, 7, 13.5, 15, 16, 17.5, 19, and 22, placed in tubes on solid CO_2 and stored at -80°C. Glands for organ culture experiments were obtained at ZT 4 to 6 and placed in culture within 60 minutes. For radiochemical *in situ* hybridization histology studies (University of Copenhagen), Sprague-Dawley and Wistar rats (Charles River, Germany) were housed for 2 weeks in a controlled lighting environment (LD 12:12). Animals were killed by decapitation at ZT6 and ZT18; their brains were removed, and immediately placed in solid CO_2 and stored at -80°C until sectioned.

Animal use and care protocols were approved by local ethical review and were in accordance with NIH guidelines, UK Home Office regulations, Health Sciences Animal Policy EU Directive 86/609/EEC (approved by the Danish Council for Animal Experiments).

Organ culture: Rat pineal glands were cultured in BGJ_b medium as previously described (56) and detailed in Supplemental Data. Glands were incubated (1 gland / well) with fresh media containing NE (1 μM) , dibutyryl cAMP (DBcAMP; 0.5 or 1 mM) or forskolin (10 μM) (Sigma-Aldrich Corp.). Following a 6 hour treatment, glands were placed in microtubes on solid CO_2.

To confirm that the glands were activated by the drugs, melatonin production in the culture media was measured by tandem mass spectroscopy as described (57), with an internal d_4-melatonin standard. The amount of melatonin produced (nanomoles/ gland/ 6 hours; mean ± standard error) for the Control, NE-treated, DBcAMP-treated and forskolin-treated groups was (number of samples) 1.4 ± 0.1 (9); 20.3 ± 1.1 (9); 9.9 ± 0.9 (9); and 15.0 ± 1.2 (9), respectively.

Microarray: For the analysis of pineal glands in experiments A and B, two sets of six pooled samples of four rat pineal glands each were prepared (three night, three day). In experiment C, four pools, each containing three glands, were prepared for each time point; as part of this experiment, single retinas and 10 mg samples of the cerebellum, neocortex, hypothalamus, liver, and heart were also obtained. Glands were also obtained from organ culture experiments in which each treatment group was comprised of three pools, each containing four glands.

Total RNA was isolated, labeled and used to interrogate Affymetrix GeneChips® as detailed in Supplemental Data.

Microarray data sets: The microarray data presented here are derived from the experiments described below (A, B and C) in conjunction with a published tissue profiling effort [Genomics Institute of the Novartis Research Foundation (GNF), Entrez Gene Expression Omnibus (GEO) dataset GDS589; http://symatlas.gnf.org (58)].

Microarray experiment A (Cardiff University) used the Affymetrix RG_U34A microarray (8,799 probe sets, 4,996 genes). Results from microarray experiment A were compared to data from the GNF database, which had been generated using the same microarray. Expression data for the following 23 Sprague-Dawley tissues and isolated cells

were used (number of samples per tissue is in parenthesis): neocortex (39), cerebellum (17), striatum (13), hippocampus (3), hypothalamus (2), pituitary (2), amygdala (10), nucleus accumbens (6), locus ceruleus (2), dorsal raphe (2), ventral tegmental area (2), pineal gland (2), dorsal root ganglion (2), cornea (2), heart (2), intestine (4), kidney (2), spleen (2), thymus (2), bone marrow (2), muscle (2), Sertoli cells (10), and endothelial cells (2).

Microarray experiment B (Cardiff University) used the RAE230A microarray (15,923 probe sets, 10,174 genes). Microarray experiment C (NICHD) used the Rat230_2 microarray (31,099 probe sets, 13,663 genes); this experiment included pineal glands and other tissues (retina, neocortex, cerebellum, hypothalamus, heart and liver) obtained at mid-day and mid-night, and glands obtained from organ culture.

Analysis of microarray results:

Night/day differences in gene expression: Affymetrix MAS5 Signal and Present Call values were stored in the NIHLIMS, a database for storage and retrieval of microarray data. The microarray data are available at the National Center for Biotechnology Information's Entrez Gene Expression Omnibus (59) and are accessible through GEO series accession number GSE12344 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12344), and at http://sne.nichd.nih.gov/microarraydata.html . Data were statistically analyzed using the MSCL Analyst's Toolbox (P.J. Munson, J.J. Barb, 2004; http://abs.cit.nih.gov/MSCLtoolbox) and the JMP statistical software package (SAS, Inc, Cary, NC; http://www.jmp.com). Affymetrix Signal values were incremented by a value of 0.1 X microarray median value, then normalized to microarray median values, and finally decimal-log transformed. This transformation is termed "Lmed," and has the desirable effect of reducing the influence of very small expression values. A one-way, two-level ANOVA testing differences between night and day was performed on the transformed data and significance [p-values, or False discovery rate (FDR) (60)] reported (see Supplemental Data Table S3). Night-day log fold changes were computed as the difference between the night and day Lmed values; in experiment C, NE/Control and DBcAMP/Control log fold change values were calculated similarly. Expression ratios are reported as linear values; negative changes are reported using the 1/X convention in which X = the Night/day ratio; i.e. a linear night/day ratio of 0.01 is displayed as 1/100. Table 1 details the expression ratios (Night/Day, NE/Control, DBcAMP/Control) of all genes with a Entrez Gene identifier and with a night/day ratio greater than 4 or less than ¼. Supplemental Data Table S3 presents the expression ratios of all probe sets with a night/day ratio greater than 2 or less than ½ .

Expression of genes in the pineal gland relative to other tissues: Gene expression in one tissue relative to expression in other tissues was defined as the relative tissue expression (rEx) value, which was calculated as the ratio of maximum expression (the highest of day or night) to the median expression of that gene in other tissues. In experiment A, median values were calculated from 23 Sprague-Dawley tissues in the GNF data base (see above; http://symatlas.gnf.org) plus the day and night pineal gland values generated in experiment A. In experiment C, the median values were calculated from the average expression levels in each of seven tissues (see above). These averages were based on single mid-day and mid-night values, except in the case of the pineal gland for which four mid-day and four mid-night values were used. The larger of two rEx values obtained using the two experiments is presented in Table 3, which contains genes with rEx values greater than 8. Supplemental Data Table S4 includes rEx values from both experiments for probe sets with rEx values greater than 2.

Comparison of results across different microarray platforms: Results from the three platforms were compared using the following mapping algorithm. The RG_U34A probe sets were first mapped to the Rat230_2 microarray using the Affymetrix ortholog map (http://www.affymetrix.com/support/technical/

byproduct.affx?product=rgu34). Exact matches for probe sets on the RAE230A are found on the Rat230_2 microarray. In cases where a probe set maps to several probe sets on another microarray, each mapped probe set pair is considered as an independent pair, resulting in multiple pairs, each sharing a common probe set. Probe sets from the different microarrays were annotated using Affymetrix annotation files (http://www.affymetrix.com/support/technical/annotationfilesmain.affx) dated November 5, 2007. Each probe set was mapped to an Entrez Gene identifier. In cases of discrepant gene identification for the probe set pair, the annotations from the most recent microarray type were used. In cases where probe sets identify two or more genes, the Gene Symbol of the first gene on the annotation file is listed, unless otherwise indicated. Summaries over multiple probe sets mapping to the same gene were calculated by taking the maximum observed ratios for night/day. The rEx values were calculated in a similar manner.

Radiochemical in situ hybridization histology: Sagittal sections of frozen rat brains were analyzed by *in situ* hybridization histology as previously described (34,61) and detailed in the Supplemental Data. Sections were hybridized with [^{35}S]-labeled 38-mer oligonucleotide probes (Supplemental Data Table S1). The sections were exposed to X-ray film or dipped into an Amersham LM-1® emulsion. The *in situ* hybridization images presented in Figure 2 are available at high resolution at http://sne.nichd.nih.gov/galleries/day_night/index.html.

qRT-PCR: For data in Figure 4 and Table 6, pools of glands were used for each time point or treatment group. cDNA was synthesized from DNase-treated total RNA and qRT-PCR results were quantitated and normalized (62) as detailed in Supplemental Data. The primers used are described in Supplemental Data Table S2.

Functional analysis: The programs used to identify clusters of genes associated with discrete functions were DAVID Bioinformatics Resources 2007 (http://david.abcc.ncifcrf.gov/), ModuleMiner(63) and NetAffx (www.affymetrix.com). The results of these analyses and common knowledge were used to generate Table 7.

Detection of cis-regulatory elements: Computational detection of enriched *cis*-regulatory elements [Position Weight Matrices: PWMs] within microarray-derived gene sets was conducted using ModuleMiner (63).

RESULTS

Microarray analysis

A large number of genes exhibit night/day differences in expression in the pineal gland. The results of analysis of night/day differences in gene expression using the RG_U34A, the RAE230A and Rat230_2 microarrays are presented in Tables 1 and Supplemental Data Table S3. To examine the degree of agreement of data obtained by these microarrays, we compared results from the RG_U34A and Rat230_2 microarray; the latter and the RAE230A microarray contain the same probe sets, representing 10,156 Entrez Gene identifiers, and did not require comparative analysis. Comparison of the expression levels of the 4,459 genes (6,392 probe sets) present on both the RG_U34A and Rat230_2 microarrays revealed the results were in excellent agreement (r = 0.44) (Supplemental Data Figure S1).

Expression of 604 genes (1,268 probe sets) exhibit a significantly greater than 2-fold change on a night/day basis (FDR < 0.20 ; Table 1 and Supplemental Data Table S3). Approximately 2,000 additional genes exhibit a smaller, but significant night/day change in expression (FDR < 0.20). These findings increase by more than 50-fold the known number of genes differentially expressed in the pineal gland. Among the 604 genes with a greater than 2-fold difference in expression, 72% increase in expression at night and that of 28% decrease. A scatter plot of the night versus day expression (Supplemental Data Figure S2A), provides an

indication of the range of night/day differences; this plot used the largest night/day difference observed with any microarray type. The amplitude of these changes varied from a downward 20-fold to an upward ~100-fold change. A set of 142 genes (209 probe sets) changes greater than 4-fold (Table 1, Supplemental Data Table S3). Among the genes listed in Table 1 are those previously reported to be night/day differentially expressed (see Introduction). Although the results obtained with the different microarrays are in excellent overall agreement, there are differences in the absolute magnitude of the night/day changes which may reflect different probe set design, biological variation, technical differences or a combination.

NE/cAMP signaling plays a dominant role in the control of night/day changes in gene expression. As noted in the Introduction, thirteen genes were previously known to be differentially expressed on a night/day basis in the pineal gland and to be controlled by NE/ cAMP signaling (1,4). To determine if additional genes exhibiting night/day differences in expression are also controlled by NE/ cAMP signaling, we used a well-established organ culture method in which glands are incubated for 48 hours, during which time nerve endings disintegrate. After 48 hours glands are treated with NE. A 6-hour treatment period was selected to approximate the time period between lights-off and mid-night sampling in the *in vivo* experiments; a dose of 1 μM NE was selected because it is known to selectively activate α-adrenergic and β-adrenergic receptors in this system (6,10,12,13,56) . Gene expression was studied using the Rat230_2 microarray (experiment C).

Approximately 98% of the probe sets that exhibited increased expression at night also exhibited increased expression following NE treatment; and, 85% of the probe sets that exhibited decreased expression at night also exhibited decreased expression following NE treatment (Supplemental Data Figure S2B; Table 1 and Supplemental Data Table S3). This finding supports the conclusion that night/day differences in gene expression in the pineal gland are due to a large degree to the release of NE from nerve terminals in the pineal gland (4).

NE activates adenylate cyclase and elevates intracellular cAMP levels in the pineal gland. Here it was found that most effects of NE were mimicked by treatment with 0.5 mM DBcAMP (Supplemental Data Figure S2C; Table 1 and Supplemental Data Table S3). 95% of the probe sets that exhibited decreased expression following NE treatment also exhibited decreased expression following DBcAMP treatment. The finding that DBcAMP treatment broadly mimics the effects of NE on gene expression provides evidence that cAMP is the primary second messenger mediating NE control of gene expression in this tissue.

Although it is apparent that NE or DBcAMP treatments change gene expression in a pattern similar to the changes seen on a night/day basis, there are striking exceptions, *i.e.* genes that exhibit marked night/day changes in expression that exhibit 10-fold lower response to NE treatment. These include *Ccl9, Cd8a, Cyp1a1, Drd4, Mfrp, Per2, Prlr, Slco1a5* and several genes that do not have Gene Symbols (Table 1).

Organ culture has marked effects on expression of a minor component of genes. To determine whether changes in gene expression are induced by organ culture itself, we compared the day values from the *in vivo* study to control values from the organ culture study (experiment C). The normalized expression levels of more than 95% of the genes were unchanged after organ culture. However, marked changes occurred in 5% of the genes, most notable were the greater than 10-fold decreases in gene expression which occurred in 49 genes (102 probe sets; Table 2), including 11 genes (24 probe sets) that decrease greater than 30-fold ; and, the 13 genes (20 probe sets) that exhibit a greater than 10-fold increase in expression (Table 2). Some of the genes that exhibit a greater than 30-fold decrease in expression are hemoglobin genes, suggesting that in some cases expression of a gene is low because blood cells that express these genes are present in the pineal gland when removed for *in vivo*

experiments but are lost from the pineal gland during culture.

Among the nine non-hemoglobin genes that exhibited the largest decrease (> 30-fold) in expression during culture in control glands, six were also highly rhythmic, suggesting day levels seen *in vivo* may reflect physiological regulation by NE and/or another factor. The decrease in expression during culture may reflect the absence of a factor that is necessary for NE-stimulation of these genes.

The highly expressed genes that characterize the pineal gland. Highly expressed genes were identified by determining the ratio of expression in the pineal gland relative to the median expression among other tissues (see Experimental Procedures), yielding rEx values. This was done using data obtained in experiment A (RG_U34A microarray) and in experiment C (Rat230_2 microarray); the median expression values were based on 23 (58) and 7 tissues, respectively. In both cases, brain tissues comprise approximately half of the tissues sampled. The calculated tissue medians are given in Supplemental Data Table S4.

This effort identified 996 genes (1,654 probe sets) with rEx values of 4 to ~300. One hundred fifty six genes (255 probe sets) had rEx values greater than 16 (Table 3; Supplemental Data Table S4).

The rEx values for the pineal gland were compared to those of six other tissues in an effort to identify other tissues strongly expressing the same genes. Only in the case of the retina was there a striking similarity in the genes with high rEx values, consistent with evidence that both tissues evolved from a common ancestral photodetector (64). Approximately 17% of the highly expressed genes (rEx > 8) in the pineal gland are also expressed in the retina at similar levels (Figure 1; Table 4 and Supplemental Data Table S5) . Among these highly expressed genes are those that encode signal transduction proteins (e.g. *Sag, Pdc, Grk1*) and genes encoding developmental and regulatory transcription factors (e.g. *Otx2, Crx, Pax6, Neurod1).*

Genes with high rEx values in the pineal gland but not the retina include genes encoding enzymes required for melatonin synthesis (e.g. *Tph1, Gch1, Ddc, Aanat, Asmt and Mat2a*). Among those with high rEx values in the retina but not the pineal gland are *Rho* and *Opn1mw*, which encode photosensitive G-protein-coupled receptors. A surprising observation was that expression of *Opn1sw*, which encodes another G-protein-coupled light receptor is 4-fold greater in the pineal gland; this is surprising because the mammalian pineal gland is not generally regarded as being directly photosensitive.

Radiochemical in situ hybridization histological analysis confirms gene profiling results. Radiochemical *in situ* hybridization analysis of sagittal brain sections (Figure 2) was used to confirm night/day differences in gene expression and to obtain a detailed anatomical analysis of areas of expression of the genes with a high rEx value from the microarray analysis; quantitation of the radiochemical labeling in the pineal gland revealed the results of histological studies were in excellent agreement with the microarray results, as regards both the degree of night/day differences in expression and rEx values (Table 5).

The rEx values provided by microarray analysis were also confirmed by the results of *in situ* hybridization: genes with high rEx values were found by *in situ* hybridization to be expressed highly or exclusively in the pineal gland relative to other brain regions on the section (Figure 2). In some cases, however, genes with high pineal rEx values, are also strongly and selectively expressed in other brain regions. For example: *Acly* is highly expressed in the habenular nucleus and in the mesencephalic raphe complex; *Dusp1* is highly expressed in the cortex, cerebellum and thalamus; *Gnb3* is expressed at moderate levels in many brain regions; *Nptx1* is expressed in the cerebellum and dentate gyrus; and, *Ttr* is very strongly expressed in the choroid plexus (Figure 2). In addition, the results of *in situ* hybridization provide examples of nocturnally elevated extrapineal expression of

specific genes, including *Per2* in the cerebellum and cortex and *Fosl2* in the cortex, this was consistently observed upon repeated examination of brain sections from all animals. Although these findings require further in-depth investigation, this is beyond the scope of the current report.

In sections that contained the pineal stalk or deep pineal gland, both structures were labeled with the same density and pattern as the superficial pineal gland. An example of this is found in the *Crx* panel (Figure 2). However, because the deep pineal gland was absent from most sections due to its small size and to differences in the plane of section, this was not seen on a regular basis.

It is highly likely that most of these genes are expressed in the dominant cell type in the pineal gland, the pinealocyte (5), as occurs with *Aanat* (Figure 3, A and B). However, it is also clear that some of the expressed genes with high rEx values may be expressed in non-pinealocytes, as demonstrated by the endothelial cell marker *Esm1,* which has an high rEx value (Supplemental Data Table S4) and is expressed in a pattern consistent with localization in cells confined to the perivascular spaces of the gland, where endothelial cells occur (Figure 3, C and D).

qRT-PCR analysis confirms results of gene profiling. qRT-PCR was used to confirm and extend *in vitro* and *in vivo* results obtained from microarrays. This effort included genes known to exhibit large night/day differences in the pineal gland which serve as references (e.g. *Aanat, Fosl2, Crem, Dio2*) in addition to genes that did not exhibit a night/day difference; 44 genes were examined. qRT-PCR confirmed rhythmic expression that had initially been indicated by microarray analysis (Figure 4), providing evidence that the two-point night/day sampling strategy is a productive and reliable approach, as a first step towards the identification of night/day rhythmically-expressed genes. In most cases, the amplitude of the rhythm was greater using qRT-PCR. The use of multiple time points in the qRT-PCR study provided a more complete profiling of the pattern of transcript abundance. Comparison of these patterns reveal differences in the timing of the peak in transcript abundance, consistent with previous reports (65,66).

In some cases there was a comparatively large variation at transition times between low and high expression (e.g. *Fosl2, Cited4),* which may reflect individual variation (Figure 4). In the case of *Ttr* and *Mfrp,* such variation does not appear to be associated with a distinct 24-hour pattern of expression; the similar pattern of variation might reflect a common biological link (i.e. regulation) not shared by the other genes examined.

qRT-PCR was also used to confirm the microarray results from organ culture studies. The results confirm the microarray results, providing additional support for the conclusion that night/day differentially expressed genes are controlled by a NE/cAMP mechanism (Table 6). The effects of forskolin were also examined; forskolin increases adenylate cyclase activity (67,68), resulting in an increase in cAMP. It was found to mimic the effects of DBcAMP and NE, providing further support for the conclusion that cAMP mediates the effects of NE.

Detection of cis-regulatory elements. Computational detection of enriched *cis*-regulatory elements (Position Weight Matrices: PWMs) obtained via submission of gene groups to ModuleMiner (63) showed that multiple, diverse, PWMs were enriched in the submitted groups (see Supplemental Data Tables S7 and S8). Individual gene groups were found to be associated with distinct collections of high-scoring PWMs. Notable enrichments of PWM families in particular gene groups included CREB (Rhythmic >8,<1/8), SP1 (Rhythmic >8), Oct1 (Retina), and Hox (Crx) (Pineal & Retina,Pineal). None of these were enriched in the Control group, which included genes with low rEx values (< 1.5) and night/day expression differences between ½ and 2; this group was, however, enriched in the Pax4 PWM. Also notable was the relative absence of PWMs of regulatory

transcription factor (TF) sites compared with basal transcriptional sites in the Pineal group.

DISCUSSION

The results of these studies provide the most comprehensive profile of the pineal transcriptome available, contributing to a more meaningful understanding of tissue function and providing new evidence of metabolic pathways and functional capacities of the pineal gland that have been overlooked or unrecognized. These advances include a greater than 10-fold increase in the number of genes known to be night/day differentially expressed in the pineal gland; in addition, this study has revealed that night/day differential expression is regulated by NE/cAMP signaling. Moreover, this effort has identified for the first time a large set of highly expressed genes that may or may not be differentially expressed on a night/day basis. The lists of night/day differentially-expressed genes and highly expressed genes provide a valuable new database for future studies of the pineal gland.

Gene expression considered in light of the cellular composition of the pineal gland. Discussion of the results of microarray studies is appropriately preceded by consideration of the organization and composition of the tissue. Pinealocytes are the dominant cell in the rodent pineal gland (~95%) and are recognized to function as a melatonin factory (5). Interstitial cells are located between pinealocytes and generally resemble brain fibrillary astrocytes. In addition, the mammalian pineal gland contains a dense vasculature composed of endothelial cells and pericytes of the capillaries in fairly large perivascular spaces (Figure 3), with a ground substance consisting of acid mucopolysaccharides. Some arterioles are also present, which adds the smooth muscle cell to the list of minor cellular components of the pineal gland. In addition, minor populations of phagocytes are located in the pineal perivascular spaces and in the parenchyma of the gland (69). Finally, T-cells and other members of the immune family of cells can be transiently present in the gland or located in a cluster just outside the gland (5).

This cellular complexity makes it clear that some of the highly or night/day differentially expressed genes may be located in cells other than the pinealocyte. This possibility is made clear by the example of *Esm1,* an endothelial cell marker which is known to be highly expressed in the pineal gland (70,71). Comparison of the patterns of expression of *Esm1* and of pinealocyte markers as revealed by *in situ* hybridization histology makes it clear that *Esm1* is not expressed in pinealocytes and is likely to mark endothelial cells. Previous studies with another gene, *Id1,* have provided reason of a different nature that also argues for the importance of consideration of the cellular localization of transcripts. These studies found that expression of *Id1* follows a daily rhythm (26) and is expressed at levels that are ~ 8-fold higher than in other tissues. *Id1* is expressed in the pineal gland at very high levels in a small population of glial-like cells and at lower levels in pinealocytes (72). Similarly, in evaluating the conditional changes in gene expression (Night/Day, NE, DBcAMP), it is clear that genes may be differentially expressed in non-pinealocyte sites and that evidence from histological studies or gene profiling of purified cell preparations or both is required to establish the site of expression of a gene of interest to provide the cellular context in which a gene is expressed.

The large number of genes that exhibit daily changes in expression in the pineal gland. An unexpected finding from this effort was the large number of genes that exhibit night/day differences in expression: more than 600 genes are differentially expressed on a night/day basis more than 2-fold, with ~70% increasing at night. The far greater number of night/day differentially expressed genes seen in this study as compared to the less than 40 genes seen in previous studies (26,55) may reflect several factors, including the larger number of probe sets interrogated by the RAE230A microarray (15,923 probe sets, 10,174 genes) and the Rat230_2 microarray (31,099 probe sets,

13,663 genes), as compared to the two platforms used in the previous studies, including the Affymetrix RG_U34A microarray (8,799 probe sets, 4,996 genes) and the Atlas Rat 1.2 cDNA Expression Array (1,176 genes). Other factors that may have contributed to the differences are the larger number of replicates in the current study, which is based on a total of ten pools of night and of day glands; previous studies used less. In addition, technical differences and differences in statistical analyses may have contributed to the number of genes detected.

A striking feature of the global change in gene expression is that many genes are differentially expressed on a night/day basis with a greater than 10-fold amplitude. This characteristic is consistent with the dedicated role that the pineal gland has in timekeeping.

It is likely that the number of night/day differentially expressed genes will grow in the future for several reasons. One is that the two point sampling (mid-day versus mid-night) used here may not have revealed daily rhythms in expression that peak closer to dawn or dusk, which might be revealed by more frequent sampling. For example, recent studies have revealed such a rhythm in expression of *Pax4* (73). In addition, the night/day differentially expressed genes identified in Table 1 only include those that have been assigned Entrez Gene identifiers and exceed a 2-fold night/day threshold. Approximately 500 of these have not been assigned Entrez Gene identifiers; and, although these may represent non-coding RNAs of unrecognized genes, some may represent unidentified genes. Accordingly, the complete annotation of the rat genome will further expand the number of genes that are expressed differentially on a 24-hour basis in the pineal gland, as will more frequent time sampling. Furthermore, the precise nature of the transcripts encoded by genes can not be reliably predicted from the results of microarray studies, because the microarray probes are based on 3' sequence; this may not detect differential splicing and/or the actions of alternative promoters, which can markedly alter the expressed transcript, as seen with *Crem, Slc15a1, Atp7b* and *Pde4b* (21,23,24,74).

It is important to note that changes in mRNA may be large and unequivocal but one can not reliably predict that such changes will be translated into changes in protein. Such a relationship is seen in the pineal gland in the case of *Mat2a, Slc15a1, Pde4b and Fcer1a (22,23,75,76)*; in these cases, the transcripts and encoded proteins appear to have similar stability. However, the daily changes in gene expression in other cases may be only slowly translated into changes in proteins over a period of days or weeks, not hours, thereby providing an integrated measure of prior levels of activity. That is, changes in the duration of the periods of expression of some genes may lead to very gradual changes in the level of the encoded protein as has been shown to be the case in the pineal gland with the *Crem* splice variant *Icer* (24,77) and the adrenergic receptor *Adra1b* (19). Likewise, posttranslational regulation can cause very rapid changes in protein, without the encoding mRNA changing as seen with *Aanat* (78,79).

Adrenergic/cAMP signaling plays a dominant role in controlling the global changes in pineal gene expression. As indicated in the Introduction, previous studies of night/day differentially expressed have provided convincing evidence that NE controls cAMP accumulation, which in turn controls gene expression. These studies have established clearly that norepinephrine is released at night in the dark and that when release is blocked, night/day differences in gene expression is blocked. Moreover, it has been shown repeatedly that the night/day differences in gene expression can be mimicked in organ or cell culture by treatment with NE, which elevates cyclic AMP in this tissue. Further, it has been established that in all cases, the effects of NE on gene expression are mimicked by elevation of cAMP or by cAMP protagonists. The results of the current study markedly expands the list of adrenergically regulated genes and in doing so demonstrates that a single regulatory signal can have a profound effect on the transcriptome of one tissue.

A previous study did not find a correlation between night/day differentially expressed genes and those induced by NE treatment (26,55,80). It is likely that this difference is due to the short NE treatment period (1 hour) used in the previous study (80). The current set of experiments used a 6 hour treatment period, which more closely reflects the period animals were in the dark in the *in vivo* studies.

As discussed above, the number of genes controlled by this signaling cascade is likely to increase because of annotation issues and also because only a single 6 hour treatment period was studied, which might not detect changes that occur rapidly and transiently; or those that are slow to develop. In addition, another reason that gene expression might not increase in some cases is the artificial conditions of organ culture, including use of a defined minimal medium. The absence of hormones and other factors critical for expression of some genes might preclude a response to NE or DBcAMP. Accordingly, it is possible that future investigations will find that NE induction of some genes may require one or more coregulating factors which are absent from the current organ culture medium. As a result, the number of genes regulated by NE would increase.

The effects of cAMP, as addressed in the Introduction, are likely to be mediated by PKA and reflect either a specific pCREB/CRE interaction or more general epigenetic mechanisms, including regulation by histone H3 phosphorylation and acetylation. Epigenetic modulation of chromatin organization could influence access of transcription factors to regulatory elements in genes. In this case, cAMP can be seen as a transcriptional regulator acting through epigenetic mechanisms that do not involve pCREB/CRE interactions.

The finding that cAMP suppresses expression of some genes may be explained by the induction of inhibitory transcription factors, as recently discussed (81) and also, along epigenetic lines, because cAMP may act to block access to a regulatory element by altering chromatin structure.

The finding that most of the genes that are differentially expressed on a night/day basis are also regulated by NE provides evidence for concluding that these genes are expressed on a circadian basis, i.e. a daily rhythm will be seen in constant darkness and does not require light/dark transitions. This conclusion is supported by the fact that the release of NE into the pineal extracellular space is controlled by the SCN. As discussed in the Introduction, the SCN/SCG/NE/cAMP regulatory system has been found to regulate a small number of genes in the pineal gland based on classical biochemical and physiological evidence. The results of the current study provide reason to conclude that all genes that have been found in the current study to be controlled by NE and DBcAMP are physiologically controlled by the endogenous circadian oscillator in the SCN and that their rhythms can be correctly described as circadian in nature.

Genes that are spontaneously up- or down-regulated in organ culture. The relative level of expression of most genes compared to total gene expression does not change remarkably after pineal glands are placed in culture. However, we discovered a subset of genes which exhibited greater than 10-fold positive and negative changes during culture. Decreased gene expression may reflect the absence from organ culture of a regulatory factor or hormone normally present in the circulation; or, of a transmitter (e.g. dopamine or neuroactive peptides) that is normally released from the sympathetic nerve endings. Gene expression may also change in response to the culture environment (95% O_2 and defined medium) or because physiologically relevant local control mechanisms do not function *in vitro.* For example, these changes could reflect the absence of interactions between pinealocytes and the vasculature.

It is of special interest to note that there was a marked decrease in the expression of one of the more important genes in the melatonin synthesis pathway, *Asmt,* which encodes the last enzyme in melatonin synthesis. This has not been reported previously. Studies with rodents have revealed that expression of this enzyme can be

regulated by adrenergic mechanisms (82,83). Other studies of this gene in Y79 cells, a human retinoblastoma-derived line, indicate that expression of the gene is controlled by 9-*cis*-retinoic acid (84). However, we have not been able to prevent the decrease in *Asmt* expression in the cultured rat pineal gland by treatment with 9-*cis*-retinoic acid, NE or DBcAMP (unpublished studies). Accordingly the factors controlling expression of this gene remain unknown.

Studies with retinoblastoma cells have also found that 9-*cis*-retinoic acid regulates expression of *Crx* and a set of genes expressed in cones (85), pointing to the need for further studies on 9-*cis*-retinoic acid and the role it plays in pineal biology. Similarly, expression of the opsin gene *Opn1sw* falls more than 50-fold in culture; its expression is controlled by thyroid signaling (86). Accordingly, it is possible that treatment of pineal glands with thyroid hormone and retinoic acid, which are known to act in concert through heterodimeric receptor complexes, may prevent some of the spontaneous large changes in gene expression that occur during culture and that media used for pineal organ cultures should be supplemented accordingly.

As mentioned in the Results section, it is also likely that the disappearance of some genes reflects the loss of blood cells normally present in the vasculature, as is probably the case for *Hba-a1* and *Hbb* which are expressed in the red blood cells.

Functional implications. Functional clustering (see Procedures) of the genes selected for inclusion in Table 7 (rhythmically expressed or highly pineal-enriched, or both) places these genes into two broad functional categories. These are, firstly, groups of genes that participate in specialized functions, and, secondly, genes that have a non-specialized, more common role in cell biology.

Specialized functional gene groups: Two of these gene groups are predictable from previous knowledge (melatonin production and phototransduction) whereas two other groups (immune response and thyroid/retinoic acid signaling) are less predictable.

Melatonin production: Most predictable are those genes that code for proteins involved in melatonin production including both enzymes that function directly in the melatonin synthesis pathway and cofactors required for these enzymes. It is also very likely that many of the genes encoding proteins dedicated to protein phosphorylation and to cAMP and calcium signaling are involved in the control of melatonin synthesis, in part through regulation of *Aanat* expression and processing of AANAT protein.

Phototransduction: Gene expression similarities between the pinealocyte and the retina have been documented before, but were limited to less than 20 genes. The current study greatly extends the number of genes known to be highly expressed primarily in pineal/retina to over 55 genes (63 probe sets). A common expression pattern reflects well-documented evolutionary relationships between the pineal and retina (54,87,88) but by revealing the real extent of pineal/retina co-expression we have freshly questioned the functional relevance of phototransduction-related genes in the mammalian pineal gland (see below). Highly expressed pineal/retina genes also include transcription factors (*Otx2, Crx, Neurod1 and Pax6*) that likely drive the common transcriptional outputs in these tissues. The work done here also led to the finding that both tissues express high levels of *Pax4,* an ortholog of *Pax6* (89). Importantly, adult expression of these transcription factor genes implies roles in maintenance of cellular phenotype, in addition to roles during development.

It is of interest to note that the expression of one opsin gene in the pineal gland is higher than that in the retina: *Opn1sw* is expressed 4-fold higher in the pineal gland as compared to the retina. In contrast, *Opn1mw* and *Rho* are expressed at 18- or >400-fold higher in the retina, respectively. This, and the finding that the pineal expresses many genes involved in phototransduction provide genetic evidence that this tissue might detect light; however, there is no evidence that the adult pineal gland has this capacity. Photodetection by neonatal pineal gland has been reported (90-92); however, the underlying mechanism involved has not been elucidated and it is not clear whether this

involves the phototransduction system that operates in the retina or another mechanism. Pineal *Opn1sw* may be functionally vestigial in the adult pinealocyte as regards detection of light. However, it is also possible that it plays a passive role in signal transduction that does not involve detection of light; for example it might influence signal transduction by binding to receptors and other proteins involved in adrenergic signaling.

Immune/inflammation response: Our identification of a large cluster of immune/inflammation-associated genes expressed in the rat pineal gland reflects similar findings in an avian species [88] and is therefore of interest. A potential, immune-related, functional specialization for the pineal gland is indicated by the presence of perivascular phagocytes that act as antigen-presenting cells (69), and also by the strong expression of *Fcera1* (a receptor dedicated to IgE signaling) and a related gene in the pineal gland (76). Further study of these genes may lead to a better understanding of the role of the pineal gland in the immune response.

Thyroid/retinoic acid signaling: Our finding of high levels of transcripts associated with thyroid/retinoic acid signaling suggests a potential functional specialization related to this signaling pathway. Previously there has been a report of effects of thyroid hormone on melatonin synthesis and a substantial body of evidence indicating that *Dio2* is night/day differentially expressed in this tissue (93-95). Together with reports of effects of thyroid hormone on retinal function (86,96-99) the accumulated evidence argues for future studies that involve specific functional interventions of this signaling pathway in both the pineal gland and retina.

Non-specialized functional gene groups:

Cellular signaling: A functional cluster of 'cellular signaling' genes derived from the genes of Table 7 is consistent, at least in part, with the evidence of adrenergic control of pineal function, which involves a broad range of signal transduction-related proteins. As indicated above, many of these are likely involved in regulating melatonin production. The high rEx of *Drd4* and known rhythmic pattern of expression (26) , points to a related role for dopamine in pineal function because dopamine is colocalized in pineal nerve processes as a precursor of NE; as such, it is likely to be released with NE. The role of dopamine in the pineal gland remains to be fully established. It is of interest that *Drd4* expression was not elevated by NE or DBcAMP in organ culture. This raises the question of how expression of this gene is regulated *in vivo*.

The current study has also highlighted groups of genes that have not received significant attention. These include genes related to prostaglandin synthesis and the lipoxygenase 15 pathway, which leads to production of hepoxilins and related compounds (100-106). The regulation and function of these pathways and their cellular sources and targets requires further investigation. Other less well-studied genes in a pineal context included the receptors for prolactin, acetylcholine, GABA, glutamate and interleukin, suggesting roles in pineal gland signaling. Acetylcholine receptor expression is consistent with anatomical evidence (5) that reveals the presence of non-sympathetic, probably parasympathetic, nerve fibers in the pineal gland of the rat and with biochemical studies which have shown the presence of both muscarinic (107) and nicotinic (108) cholinergic receptors. Further, it has been shown that a cholinergic input to the rat pineal gland causes the release of glutamate, which has been reported to act *via* glutamate receptors on the pinealocyte membrane to inhibit melatonin synthesis (109,110).

Genes dedicated to small molecule biology: Included amongst this cluster of genes is the taurine transporter *Slc6a6*, perhaps explaining the high concentration of taurine in the pineal gland (111); this gene is also highly expressed in the retina, another tissue with a high concentration of taurine (112). Genes that regulate metal homeostasis are also clustered here. Zn^{++} is essential for the synthesis of melatonin, because it is required by Gch to generate biopterin (113,114), the cofactor for Tph1, the first enzyme in melatonin synthesis. Another essential role of

metals of special relevance to pineal physiology is Sn+, which is essential for T3 signaling because it is required by Dio2 (115).

Cell:cell and cell: extracellular matrix contacts: Genes encoding proteins involved in cell:cell and cell: extracellular matrix contacts represent another group of genes that have not been well studied in the context of the pineal gland. This includes several members of the cadherin family, which form homophilic Ca^{++}-dependent associations. Knowledge of the site of expression of these genes may be of practical utility in identifying, purifying and immobilizing populations of cells recovered from the pineal gland.

Circadian clock genes: Generally absent from the list of strongly rhythmic or highly expressed (Table 7) genes are circadian clock genes, exceptions being *Per2* and *Rorb.* Expression levels of *Arntl, Clock, Per1, Per2, Per3, Cry1, Cry2* and *Arnt* are less than 4-fold the median tissue level of expression. Reports in the literature have established that these genes are expressed in the pineal gland, some following a 24-hour pattern (116-119). Their absence from the lists of highly expressed or highly rhythmic genes may reflect a relative unimportant role that the circadian clock system plays in the mammalian pinealocyte in generating daily rhythms. The strong rhythm of *Per2* may reflect a related role in biological time keeping, in that it might influence the dynamics (intensity, duration) of the responses of genes with E-boxes, which includes *Aanat* (120,121).

Cis-regulatory elements. A question raised by our finding of large groups of pineal-specific and night/day differentially expressed genes is whether these groups of genes share common transcriptional regulatory DNA sequences. This question was addressed using ModuleMiner (63) using multiple groups of genes as inputs. In general, the results of this effort did not provide an indication that pineal specificity or night/day differences can be explained by the presence of a few dominant regulatory elements. This may relate to inherent limitations of this analysis, which include the analysis of only 10 kb 5' to the transcription start site and filtering which eliminates genes that do not exhibit human-mouse conservation of regulatory elements. Conversely, it is possible that rhythmic or relatively high expression of genes in the pineal gland reflects multiple parallel/hierarchical regulatory cascades of such complexity that they would be impenetrable to this mode of analysis. However, some of the results of this analysis (see Supplemental Data Table S7) are worthy of further consideration.

Enrichment of a CREB family PWM in the group of highly rhythmic genes (Rhythmic >8, <1/8) is consistent with known, cAMP-driven, mechanisms of gene regulation in the pineal gland, as discussed in the Introduction. The NE/ cAMP cascade is evident from the results of the experiments presented here, in which gene expression was broadly enhanced by treatment with DBcAMP, a stable cAMP analog; with forskolin, an activator of adenylate cyclase; or, with NE, which elevates cAMP in the pineal gland. Conversely, the absence of a similar enrichment in less highly rhythmic genes suggests the presence of alternative mechanisms. The enrichment of SP1 PWMs in moderately rhythmic and highly rhythmic groups is interesting in this context and may warrant further investigation because a previous study has indicated a role for SP1 in pineal gene rhythmicity (122).

Surprisingly, we did not observe widespread enrichment of Hox family PWMs across the tissue-specific (pineal and retina) groups of genes; this is surprising because the Crx and Otx transcription factors are known to regulate pineal- and retina-specific gene expression, as discussed in the Introduction (31,32,123). Our results show the Crx PWM to be enriched only in the combined submission of Pineal only & Pineal/Retina groups. This finding may indicate a more limited (pineal and retina related) role for Hox family-related mechanisms than is currently believed. With respect to the retina, our analysis has revealed strong enrichment of another PWM family (Oct1) which is recognized as one PWM family/transcription factor that is associated with the visual perception GO term (GO:0007601; Matbase, Genomatix). With respect to the pineal, an

intriguing finding of the ModuleMiner analysis was the relative absence of conserved regulatory transcription factor sites in the 'Pineal only' group. This is an interesting result in which ModuleMiner detects only a few enriched sequences and these are primarily either core promoter elements such as initiator and cap or CEBP, all of which are among the most common sites found in eukaryotic promoters (124). This finding taken together with the (Pineal Only & Pineal/Retina) result is suggestive of common *cis*-regulatory rules for pineal/retinal tissues but the absence of a pineal-only mechanism – indicating either a novel *cis*-, or *trans*- (e.g. miRNA) level of control not detected by this analysis, or that the pineal-specific cascade is controlled by multiple unrelated mechanisms.

Another surprising finding of the ModuleMiner analysis was the enrichment of Pax4 sites within the control group of pineal genes – genes expressed in this tissue that are neither rhythmic nor highly expressed relative to other tissues. Given the high relative expression of Pax4 in the rat pineal gland (73); this may point to a negative mode of regulation in which the 'control' gene group is suppressed *via* a Pax4-related mechanism. In this respect, previous studies have provided evidence of repressive actions of Pax4 (125).

Final statement: The results of these studies provide investigators with a rich and comprehensive genetic profiling of the rodent pineal gland and should provide a sound foundation for future investigations of this tissue focused on the factors controlling developmental and rhythmic gene expression. The evidence provided here also provides a basis for future research on the role of thyroid/retinoic acid signaling in the pineal gland and on the role of this tissue in the immune/inflammation response. The finding that most genes that exhibit daily changes in expression are regulated by NE/cAMP signaling raises intriguing questions regarding the mechanisms involved, especially the role of epigenetic events. Analysis of the factors regulating these large changes in gene expression and expression of genes at relatively high levels in the pineal gland may be of interest to investigators studying these genes in other tissues, especially the retina. The indication from the results of analysis of *cis*-regulatory elements that transcript abundance in the pineal gland involves PWMs which have not previously appeared in the pineal literature opens new doors to the analysis of the molecular control of gene expression in this tissue.

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FOOTNOTES

[1] One transcript isoform of *Crem*, termed *Icer*, is known to be highly rhythmic in the rat pineal gland. Therefore, throughout the text, when *Crem* is mentioned in the context of the pineal gland, the term refers to the *Icer* isoform.

[2] Where numbers of genes in various categories are given, this refers to probe sets that have been annotated with Entrez Gene identifiers by Affymetrix as of November 5, 2007, and updated manually as of June 15, 2008. The Gene Symbols that are used have been taken from Entrez

Gene; associated Gene Titles and Entrez Gene identifiers are given in Supplemental Data Tables S3, S4 and S5. Gene Symbols beginning with LOC, RGD or MGC are not included in the tables in the text; they are included in the Supplemental tables.

The abbreviations used are: Rat230_2 (Affymetrix GeneChip® Rat Genome 230 2.0); RAE230A (Affymetrix GeneChip® Rat Expression Array 230A); RG_U34A (Affymetrix GeneChip® Rat Genome U34A); cAMP, cyclic adenosine monophosphate; DBcAMP, dibutyryl cAMP; CREB, cAMP response element binding protein; pCREB, phosphor-CREB; CRE, cAMP response element; cGMP, cyclic guanosine monophosphate; FDR, false discovery rate; qRT-PCR, quantitative real time polymerase chain reaction; PWM, position weight matrices; SCG, superior cervical ganglia; SCN, suprachiasmatic nucleus; NE, norepinephrine; PKA, protein kinase A; ZT, Zeitgeber time; Hab, habenula; ic, inferior colliculus; mhn, medial habenular nucleus; Raphe, dorsal raphe nucleus; sc, superior colliculus.

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FIGURE LEGENDS

Fig 1. Pineal rEx vs Retina rEx. This figure demonstrates that a subset of genes is predominantly expressed in the pineal gland or retina, or both. Data are based on results obtained in experiment C. The probe sets for the entire microarray are represented by 5% density contours (95% of probe sets are within the outermost contour line). Only symbols representing probe sets with rEx values greater than 8 for either the pineal gland or retina (approx 1% of 31,099 probe sets on the Rat230_2 microarray) are shown; the remaining probe sets are not highly expressed in either tissue relative to other tissues. The plotted probe sets fall into three sectors termed Pineal, in which 218 genes (256 probe sets) are highly expressed primarily in the pineal gland; Pineal,Retina, in which 55 genes (63 probe sets) are highly expressed at similar levels in both the pineal gland and retina; and, Retina, in which 93 genes (109 probe sets) are highly expressed primarily in the retina. The genes represented by these probe sets are listed in Table 4; Supplemental Data Table S5 contains a detailed description of these genes and other unannotated probe sets. An interactive version of this figure that identifies each symbol is available at http://sne.nichd.nih.gov/data/interactive.html.

Fig 2. Radiochemical *in situ* hybridization images. Each panel contains autoradiographs prepared from sections of rat brains through the pineal gland. The sections on the left are from animals killed during the day and those on the right are from animals killed during the night. The sections were incubated with antisense probes identified in the bottom left hand corner of the Day image. Probes are detailed in Supplemental Data Table S1. The results of quantitation of the

signal strength of the pineal labeling appear in Table 5. For further details see Experimental Procedures. Hab=habenula, ic=inferior colliculus, mhn=medial habenular nucleus, Raphe=dorsal raphe nucleus, sc=superior colliculus. These figures are available in high resolution at http://sne.nichd.nih.gov/image_gallery.html.

Fig 3. Staining of pinealocytes and endothelial cells in the pineal gland. **A.** Image presenting Aanat-labeling of pinealocytes. Dark field photomicrograph of radiochemical *in situ* hybridization of a part of the superficial pineal gland of the rat with an antisense probe binding to mRNA encoding AANAT. The section was dipped in a photographic emulsion and developed after exposure; the grains are seen using dark field visualization as white dots in the emulsion. Dense labeling is seen above the pineal parenchyma with no labeling of the perivascular space (per vasc). **B.** The same section shown in Panel A in transmitted light. Arrows indicate the perivascular space, which is not labeled in panel A. Cresyl violet counterstaining. **C.** Labeling of the perivascular spaces: Endothelial labeling image. Dark field photomicrograph of radiochemical *in situ* hybridization of the superficial pineal with an antisense probe binding to mRNA encoding Esm1. Note the labeling of the perivascular spaces (arrows). **D.** High power photomicrograph taken in transmitted light of part of the superficial pineal showing *Esm1* labeling of the perivascular cells and only few grains above the pinealocytes. Cresyl violet counterstaining. Bars: A, B, and D = 20 μm. C = 50 μm. The probes used are described in Supplemental Data Table S1.

Fig 4. qRT-PCR analysis of transcripts that are night/day differentially expressed or have high rEx values, or both. The lighting cycle is represented at the bottom of each column. Transcripts are identified by Gene Symbol. Each value is the mean ± standard error of 3 determinations. Values were normalized to *Actb, Gapdh, Hrpt1 and Rnr1*. A single asterisk (*) identifies statistically significant rhythmic patterns of gene expression ($P < 0.01$) based on log transformed raw values analyzed by one-way ANOVA in JMP. For technical details, see Experimental Procedures and Supplemental Data.

Table 1. Differential expression of genes in the pineal gland. Expression ratios listed under Night/Day (N/D) are the highest values obtained in experiments A, B or C (see Experimental Procedures). Expression ratios listed under NE/C (NE/Control) and DBcAMP/C (DBcAMP/Control) are from experiment C. Decreases in expression are indicated by the 1/X convention, indicating that expression was lower by a factor of X. Section A includes genes that exhibited a >8 or <1/8 fold Night/Day difference in expression; Section B includes genes with a 4- to 8-fold or ¼ to 1/8 Night/Day difference in expression. Supplemental Data Table S3 (http://sne.nichd.nih.gov/data/table_s3.xls) is an expanded version of this table, and includes genes with a 2- to 4-fold or 1/2 to ¼ -fold Night/Day difference in expression, Gene Titles, probe set numbers and Entrez Gene identifiers. *, predicted gene.

Section A. Genes with N/D ratios > 8 or < 1/8

Gene Symbol	N/D ratio	NE/C ratio	DBcAMP/C ratio
Aanat	92.9	46.8	69.3
Arpp21	11.8	1/ 2.1	1.3
Atp7b	12.1	16.1	17.4
Ccl9	1/ 16.5	1/ 1.6	1.0
Cd24	12.4	3.2	5.0
Cebpb	9.6	2.3	1.8
Cited4	12.2	1.5	3.1
Crem	37.0	10.3	17.4
Cyp1a1	9.1	1.0	1/ 1.2
*Dclk3**	20.2	9.1	15.1
Dio2	8.8	6.9	14.3
Drd4	29.5	3.5	2.2
Dusp1	48.8	7.9	9.2
Errfi1	10.6	1.9	3.6
*Etnk1**	9.9	2.2	3
Fcer1a	15.2	28	35.2
Fgfr1	8.1	1.0	1.2
Fst	19.6	3.4	2.6
Gdf15	12.4	2.1	3.9
Hs3st2	1/ 21.4	1/ 8	1/ 5.9
Hspa1a	9.7	1.5	1/ 1.1
Irak2	14.5	4.5	5.6

Gene Symbol	N/D ratio	NE/C ratio	DBcAMP/C ratio
Irs2	15.0	6.3	6.7
Man2a1	9.7	2.5	3.7
*Mfrp**	12.1	1/ 1.8	1/ 1.4
Mt1a	12.6	1.1	2.8
Nap1l5	18.5	4.4	12.5
nod3l	19.8	4.7	12.6
Nr4a1	8.0	3.4	6.5
Nr4a3	8.2	7.7	2.7
Pde10a	12.3	7.9	8.0
Per2	13.4	1.2	1.1
Prlr	9.8	1.1	1.1
Ptpn21	1/ 8.1	1/ 3.5	1/ 3.9
Ribc2	26.3	5.9	22.3
Slc15a1	74.3	19.6	39.3
Slc6a17	9.3	6.5	4.2
Snf1lk	26.0	8.2	15.3
Sostdc1	9.8	1.5	1.3
St8sia5	16.9	1.3	1.9
Syt4	9.0	1.8	5.8
*Wnt10a**	9.7	9.2	7.0
Zrsr1	12.7	2.2	5.3

Table 1, Section B Genes with N/D ratio 4 to 8 or 1/4 to 1/8

Gene Symbol	N/D ratio	NE/C ratio	DBcAMP/C ratio
Abca1	6.7	1.8	3.3
Ace	6.2	1/ 1.7	1.3
Adcy8	5.4	1/ 1.5	2.6
*Ankrd52**	4.8	1.9	2.0
Anp32a	4.5	1.0	1.8
Atp2b3	6.0	1.1	1/ 1.3
Bex1	4.5	1.6	1.6
Btg2	5.0	1.9	1.5
Cacna1g	4.4	5.2	9.3
Camk1g	7.6	4.8	6.8
*Ccrl2**	4.1	1.1	1.4
Cd8a	7.9	1/ 1.2	1/ 1.9
*Cfl2**	1/ 4.2	1/ 1.3	1/ 1.2
*Chst2**	4.5	2.2	7.8
Cip98	4.0	4.8	3.4
Col5a3	1/ 6.3	1/ 1.5	1.1
Coq10b	5.4	2.5	2.7
Cry2	4.7	1.5	1/ 1.2
Dclk1	6.6	2.1	2.4
Ddit3	4.2	1.0	1.2
Dnm2	4.4	3.1	4.0
*Dos**	7.1	2.8	4.4
Dsc2	4.5	1/ 1.8	1.1
Dscr1	6.2	4.0	4.2
Egf	1/ 4.1	1.0	1/ 2
Emd	7.2	1.4	1.5
Eomes	7.4	4.5	3.7
Fdx1	5.7	10.0	27.5
Folr1	4.0	1/ 1.2	1/ 1.4
Fosl2	4.2	3.0	4.4
Gadd45b	4.7	1/ 1.9	2.1
Galnt14	1/ 5.5	1/ 1.2	1/ 1.7
Galntl1	5.3	1.6	2.6
Gclm	4.7	1.0	1.1
*Gem**	4.5	2.9	3.2
Gls	4.9	1.8	1.8
Gls2	5.6	1.3	2.0
Glt8d3	4.1	2.1	2.9
Grm1	6.0	2.9	1.1
Hcrtr1	7.9	1.6	3.0
Hhip	7.4	3.1	3.7
Hspa1b	6.8	1.5	1.3
Hsph1	4.3	1.4	1.9
Igf1r	6.7	3.6	3.5

Gene Symbol	N/D ratio	NE/C ratio	DBcAMP/C ratio
Igfbp2	4.0	1/ 1.1	1/ 1.2
Kcnab2	4.2	1.3	2.3
Kctd3	7.4	2.1	4.3
Lamb3	6.3	3.7	4.2
Lcn7	6.5	2.9	8.1
Lphn2	4.0	1.9	1.6
Lrrn3	7.1	5.9	4.5
Lxn	5.4	1.1	2.7
Mat2a	5.8	3.2	6.1
Mcam	4.8	2.0	3.6
Mina	1/ 4.0	1/ 1.7	1/ 2.1
Mtac2d1	5.2	3.3	3.3
Muc4	1/ 6.9	1.2	1/ 2.2
Nphp4	7.3	5.2	4.7
Padi4	4.4	2.0	3.0
*Parvb**	4.1	1.4	1.3
*Pcdh1**	4.1	2.0	3.1
Pde4b	6.7	3.2	4.1
Pde4d	5.8	2.4	1.3
Pde8b	4.0	4.7	4.7
Plagl1	5.3	6.9	2.3
Ptch1	4.3	2.3	5.8
PVR	7.6	1.6	1.7
Qscn6	4.5	1.7	2.7
Rab3ip	4.5	1.5	1.7
Reln	4.2	1/ 1.3	1/ 1.1
*Rhobtb3**	4.2	1/ 1.1	1.7
Rock2	4.7	1.2	1.4
*Scrt1**	4.1	1.5	2.4
Sgk	1/ 4.6	1/ 3.2	1/ 1.4
Slc12a2	4.6	1.5	1.2
Slc17a6	4.1	4.3	5.5
Slc30a1	5.4	1/ 1.6	1.9
Slco1a5	5.0	1/ 2.6	1/ 3
Sorl1	1/ 5.0	1.8	1/ 1.2
St8sia3	1/ 4.5	1/ 1.4	1/ 1.5
*Tbc1d1**	6.1	3.1	2.7
Tfpi2	1/ 5.5	1.6	1/ 1.2
Tjp2	4.7	1.6	1.6
Tmed1	1/ 5.4	1.1	1/ 1.4
Top1	4.3	1/ 1.1	2.5
Tyro3	6.1	1.6	4.3
Vof16	1/ 4.2	1/ 2.4	1/ 2.1
Wdr89	1/ 4.3	1/ 1.2	1/ 2
*Xpot**	4.2	2.3	4.7

Table 2. Genes expressed 10-fold higher or lower following organ culture. Genes were identified from data generated in experiment C. Unidentified genes are not included in the table. For further details see Experimental Procedures. *, predicted gene.

Gene expression following organ culture	Gene Symbol
Higher (> 30-fold)	*Mmp3, Spp1*
Higher (10- to 30-fold)	*Akr1b8, Ccl2, Ccl20, Fcgr3, Fxyd2, Fzd1, Gpnmb, Hmox1, Igfbp3, S100a4, Wnt2*
Lower (10- to 30-fold)	*Alas2, Arr3, Asmt, Atp1a2, Atp2b3, Car14*, Ccl9, Cd74, Cd8a, Cdkn1c, Cirbp, ClicLIC6, Cln6*, Cml5, Cyp1a1, Enpp2, Folr1, Gjb6, Gpc3, Grk1, Grm1, Guca1a*, Il17re, Kcne2, Mdk, Mfap5*, Mpp4, Nphs1, Nrxn3, Ogn*, Ptgds, Reep6, RT1-Da, Sag, Slc24a1, Slco1c1, Spink4, Vtn, Vwf, Wasl*
Lower (>30-fold)	*Cox8h, Defb24, Drd4, Hba-a1, Hbb, Igfbp2, Mfrp*, Opn1sw, Prlr, Sostdc1, St8sia5*

Table 3. Genes highly expressed in the pineal gland relative to other tissues. Genes were identified from data generated in experiments A and C. Gene Titles, Entrez Gene identifiers and individual rEx values obtained in each experiment are presented in Supplemental Data Table S4 (http://sne.nichd.nih.gov/data/table_s4.xls); Table S4 also contains genes with rEx values 4 to 8. For details regarding calculation of rEx values see Experimental Procedures. *, predicted gene.

Pineal rEx	Gene Symbol
>16	*A2m, Aanat, Abca1, Abhd14b, Adra1b, Adrb1, Aipl1, Alox15, Arhgap24, Arr3, Asl, Asmt, Atp7b, Ca3, Cabp1, Cacna1f, Camk1g, Ccl9, Cd1d1, Cd24, Cdh22, Chga, Chrna3, Chrnb4, Cnga1, Cngb1, Cntrob*, Col8a1*, Cplx3, Cpt1b, Crem, Crocc*, Crtac1, Crx, Ctsc, Cyp1b1, Dclk3*, Ddc, Defb24, Drd4, Dusp1, Efemp1, Egflam, Esm1, Eya2, Fcer1a, Fdx1, Fkbp4, Fkbp5, Frmpd1*, Fst, Fzd4, Gch, Gdf15, Gem*, Gnat2*, Gnb3, Grk1, Guca1a*, Hs3st2, Hspa1a, Hspa1b, Hspb1, Igfbp6, Impg1, Impg2, Irak2, Irs1, Isl2, Ka15, Kcne2, Kcnh6, Kcnj14, Krt1-19, Lamp3, Lgals1, Lgals3, Lhx4*, Lix1*, Lpl, Lrrc21, M6prbp1, Map4k1*, Mat2a, Mcam, Me2*, Miox, Mitf, Morn1, Mpp3, Mpp4, Mtac2d1, Mx2, Ncaph, Neurod1, Nphp4, Nphs1, Nptx1, Opn1sw, Osap, Otx2, Padi4, Pax4, Pax6, Pcbd1, Pcdh21, Pdc, Pde4b, Pla2g5, Plscr1, Rbp3, Rds, Ribc2, Rom1, Rorb*, Rxrg, Sag, Scn7a, Serping1, Slc12a5, Slc15a1, Slc17a6, Slc24a1, Slc30a1, Slc39a4*, Slc6a6, Snap25, Snf1lk, Sorl1, Spink4, Stk22s1, Sv2b, Tm7sf2, Tph1, Ttr, Tulp1*, Unc119, Vof16*
8 to 16	*Accn4, Acsl1, Acvr1, Adam2, Ak3l1, Als2cr4*, Ampd2, Anp32e, Anpep, Atp1b2, Atp6v1c2, Baiap2l1, Bmp6, Bzrp, Cacna1h, Ccdc125, Ccl2, Ccl6, Ccnd2, Cd63, Cd74, Cd8a, Cebpb, Cfd, Cflar, Chst2*, Cip98, Col15a1, Col1a1, Cr16, Crcp, Cyp1a1, Dcn, Depdc7, Dhrs8, Dnajc12, Dnm2, Dnm3, Dpt*, Dsc2, Dscr1, Epb4.1, Errfi1, Etnk1*, Exoc5, F5, Farp2*, Fosl2, Foxd1, Frmd4b, G0s2, Gabrr1, Gale, Galnt4, Galntl1, Gla, Gls, Gmds, Gnas, Grm1, Hcn1, Hk2, Hsd3b7, Hspb6, Id1, Ifitm3, Igfbp2, Igsf1, Igsf4a, Il13ra2, Il17re, Irf7, Itgb2, Kctd14*, Kctd3, Kit, Klhl4, Lad1*, Lama2*, Lamb1*, Lmbr1l, Lmod1*, Lnx1*, Lox, Loxl1, Lrrc8e, Lum, Lxn, Mad2l2, Mak, Mak10, Man2a1, Mapk6, Msrb2, Msx1, Mt1a, Muc4, Mylk*, Myo5b, Nacad, Nr4a1, Nradd, Nrap*, Nup107, Oasl1, Orai1, Pcbp3, Pde10a, Pde6b*, Pdp2, Pgam2, Pgm1, Pid1, Pik3r3, Pla2g1b, Pla2r1*, Plcd1, Plcd4, Postn*, Pqlc1, Prkar2b, Prkca, Prtg, Psph, Ptgis, Ptms, Ptprn, PVR, Qscn6, Rab3c, Rarres1, Rasgrf2, Rax, Rere, Resp18, Rnase1, Rreb1*, RT1-Aw2, Rtbnd*, Sall1*, Sema3a, Slc12a2, Slc19a2, Slc1a5, Slc25a10, Slc47a1, Slc4a2, Slc7a6*, Slco4a1, Sod3, Spint2, Svop, Tagln2, Tcn2, Tex14*, Timp1, Tmepai*, Tnfrsf9, Ugdh, Wnt10a*, Zmat2, Zrsr1*

Table 4. Genes highly expressed in the pineal gland and/or the retina. Genes were identified from data generated in experiment C, and represent the symbols shown in the corresponding sectors in Figure 1. Supplemental Data Table S5 (http://sne.nichd.nih.gov/data/table_s5.xls) contains the Gene Titles, Entrez Gene identifiers and the rEx values for individual genes. Table S5 also includes Entrez Gene identifiers for unidentified sequences (i.e. ESTs) not included here. For details regarding the calculation of rEx values see Experimental Procedures. *, predicted gene.

Group	Gene Symbol
Pineal	*A2m, Aanat, Abca1, Abhd14b, Accn4, Acvr1, Adam2, Adra1b, Adrb1, Alox15, Als2cr4*, Ampd2, Anp32e, Arhgap24, Asl, Asmt, Atp6v1c2, Atp7b, Baiap2l1, Bzrp, Ca3, Cabp1, Cacna1h, Camk1g, Ccdc125, Ccl2, Ccl6, Ccl9, Ccnd2, Cd1d1, Cd24, Cd63, Cd8a, Cfd, Cflar, Chga, Chrna3, Chrnb4, Chst2*, Cip98, Cntrob*, Col15a1, Col8a1*, Crcp, Crem, Crtac1, Ctsc, Cyp1a1, Dclk3*, Dcn, Ddc, Defb24, Depdc7, Dhrs8, Dnajc12, Dpt*, Dsc2, Dscr1, Esm1, Etnk1*, Eya2, F5, Farp2*, Fcer1a, Fdx1, Fkbp5, Fosl2, Frmd4b, Fzd4, G0s2, Gale, Galnt4, Galntl1, Gch, Gdf15, Gem*, Gla, Gls, Gmds, Gnas, Hs3st2, Hsd3b7, Hspb1, Ifitm3, Igfbp6, Igsf1, Il13ra2, Il17re, Irak2, Irf7, Irs1, Isl2, Itgb2, Ka15, Kcne2, Kctd14*, Kctd3, Kit, Klhl4, Krt1-19, Lad1*, Lama2*, Lamb1*, Lgals1, Lgals3, Lhx4*, Lix1*, Lmbr1l, Lmod1*, Lnx1*, Lpl, Lrrc8e, Lum, Lxn, M6prbp1, Mad2l2, Mak10, Map4k1*, Mat2a, Mcam, Me2*, Miox, Mitf, Morn1, Mpp3, Msrb2, Mt1a, Mtac2d1, Muc4, Mx2, Mylk*, Myo5b, Nacad, Ncaph, Nphp4, Nphs1, Nptx1, Nradd, Nrap*, Nup107, Oasl1, Padi4, Pcbd1, Pde10a, Pde4b, Pgam2, Pgm1, Pid1, Pik3r3, Pla2g1b, Pla2g5, Plcd4, Plscr1, Postn*, Pqlc1, Prkar2b, Prtg, Psph, Ptgis, Ptprn, PVR, Qscn6, Rarres1, Rasgrf2, Rere, Resp18, Ribc2, Rnase1, Rorb*, Rreb1*, Rxrg, Sall1*, Scn7a, Sema3a, Serping1, Slc15a1, Slc17a6, Slc19a2, Slc1a5, Slc25a10, Slc39a4*, Slc47a1, Slc7a6*, Snf1lk, Sod3, Sorl1, Spink4, Tagln2, Tex14*, Tm7sf2, Tnfrsf9, Ugdh, Vof16, Wnt10a*, Zmat2, Zrsr1*
Pineal, Retina	*Aipl1, Arr3, Cacna1f, Cnga1, Cngb1, Cplx3, Crocc*, Crx, Drd4, Egflam, Frmpd1*, Gabrr1, Gnat2*, Gnb3, Grk1, Guca1a*, Hspa1b, Impg1, Impg2, Kcnh6, Kcnj14, Lamp3, Lrrc21, Mak, Mpp4, Neurod1, Opn1sw, Osap, Otx2, Pax4, Pcbp3, Pcdh21, Pdc, Pde6b*, Pla2r1*, Rax, Rbp3, Rds, Rom1, Rtbnd*, Sag, Slc24a1, Slc6a6, Slco4a1, Stk22s1, Tulp1*, Unc119*
Retina	*Abca4*, Agpat3*, Arf4l*, C1ql2, Calb2, Capn3, Cart, Cav, Cbln2, Cds1, Chrna6, Cirbp, Col11a1, Col2a1, Col5a3, Col9a1, Cryaa, Cryba1, Cryba2, Cryba4, Crybb1, Crybb2, Crybb3, Cryga, Crygb, Crygd, Cryge, Dapl1, Dkk3, Elovl4*, Fscn2*, Glra2, Gnat1*, Gnb1, Gpsm2, Gsg1, Hk2, Hmx1*, Igsf9*, Isl1, Kcnb1, Mab21l1, Mdm1, Msh5, Ng23, Opn1mw, Opn4, Pax6, Pcdh8, Pcp2, Pde6a*, Pde6h, Ppap2c, Prom1, Rcvrn, Rdh11, Rdh12*, Reep6, Rfrp, Rgr*, Rho, Rhpn1*, Rp1h, Six6*, Smarcd1*, Sncg, St6galnac2, Stx3, Susd3*, Synpr, Tbc1d12*, Tbx2*, Tcfap2a*, Tcfap2b*, Tmem136, Txnl6*, Vip, Vipr2, Wdr89*

Table 5. Quantitative analysis of the *in situ* hybridization signal in the pineal gland. The antisense probes used appear in Supplemental Data Table S1. Quantitation was done as described in the Experimental Procedures; results are given as the mean ± SE. *, $p < 0.01$ significant difference between night and day *in situ* hybridization signal.

Gene Symbol	Microarray		*In situ* hybridization		
	Night/Day[a]	rEx[b]	Day (dpm/mg tissue)	Night (dpm/mg tissue)	Night/day
Aanat	92.9	196.0	13.4 ± 1.1	3059.2 ± 183.5	227.8*
Acly	1.3	6.9	386.8 ± 63.0	722.0 ± 148.5	1.9 *
Alox15	1.7	324.0	924.5 ± 64.2	927.0 ± 79.7	1.0
Asmt	1.3	198.0	1737.5 ± 107.8	2156.4 ± 170.8	1.2 *
Atp7b	12.1	17.9	4.8 ± 0.9	424.8 ± 55.3	87.6 *
Crem	37.0	167.7	40.2 ± 1.6	1277.4 ± 240.9	31.8*
Crx	2.2	151.1	1277.2 ± 79.0	1411.0 ± 333.7	1.1
Dusp1	48.8	85.8	9.0 ± 0.8	26.5 ± 7.4	3.0*
Esm1	1.9	198.7	134.5 ± 16.7	196.7 ± 20.5	1.5*
Fosl2	4.2	8.1	15.8 ± 1.1	79.9 ± 26.8	5.1*
Gch1	1.3	236.4	980.8 ± 67.1	965.9 ± 82.7	1.0
Gnb3	1.0	101.8	3262.5 ± 309.1	3989.8 ± 228.1	1.2*
Mt1a	12.6	12.2	90.8 ± 14.4	1031.3 ± 247.1	11.4*
Nptx1	3.5	48.9	86.5 ± 5.2	543.1 ± 24.7	6.3*
Pdc	1/ 2.7	359.4	566.7 ± 53.4	170.9 ± 18.7	1/3.3*
Per2	13.4	5.1	19.5 ± 2.5	37.2 ± 1.8	1.9*
Rorb	3.4	32.9	34.7 ± 4.7	170.8 ± 32.8	4.9 *
Tph1	1.2	259.4	3092.3 ± 16.7	3049.5 ± 36.0	1.0
Ttr	1.5	65.1	1476.9 ± 138.7	2362.0 ± 291.8	1.6*

a: Taken from microarray data in Table 1

b: Taken from microarray data in Table 3 and Supplemental Data Table S4.

Table 6. Effects of norepinephrine (NE), dibutyryl cyclic AMP (DBcAMP) or Forskolin on transcript abundance in cultured pineal glands. Glands were cultured for 48 hours prior to the initiation of the indicated treatment. Treatment duration was 6 hours. Transcript abundance was measured by qRT-PCR and normalized as described in the Experimental Procedures. Each value is based on results obtained from 3 pools of 3 glands and given as a treatment/control ratio. A more extensive version of this table, including absolute values of the mean ± SE, is available in Supplemental Data Table S6. * p<0.01. # , genes used for normalization.

Gene Symbol	NE / Control	DBcAMP / Control	Forskolin / Control
Aanat	143.7*	284.8*	96.2*
Abca1	2.43*	4.21*	2.26*
Acly	1/1.27	1.34	1.52
Actb#	1/1.20	1/1.17	1/1.22
Alox15	1.14	1.62	1.59
Asmt	1.11	1.31	1.80
Ccl9	1/3.27	1/3.50	1/10.49
Cd8a	1/1.30	2.45	3.78
Cebpb	2.10	4.78*	2.40
Cited4	50.1*	84.1*	49.1*
Crem	31.6*	32.5*	27.6*
Ddc	1/1.29	1.01	1/1.07
Dio2	3.92*	10.8*	11.0*
Drd4	14.0*	41.9*	20.1*
Dusp1	13.7*	18.7*	5.86*
Egr1	1.31	1/1.03	1/1.61
Esm1	1/1.09	3.32*	2.42*
Fcer1a	3.92*	10.8*	11.0*
Fosl2	3.09*	4.74*	2.91*
Galnt14	1/3.79*	1/ 2.16 *	1/3.01*
Gapdh#	1.00	1.32	1.00
Gch1	1.25	1.18	1/1.01
Hhip	16.9*	36.5*	59.0*
Id1	2.36	2.98*	1.97
Id3	1.05	1.17	1/1.26
Il18	1.36	1.56*	1.46
Kpna2	1/1.46	1.20	1.23
Lta	1.36	2.06	1.82
Map3k5	1.20	2.45*	2.17
Mat2a	9.12*	11.0*	6.78*
Mfrp	1/9.71	1.46	1/6.67
Neurod1	1.11	1.13	1.27
Npy1r	1/3.81*	1/2.69*	1/2.01
Nr4a2	1.76	5.07*	2.42
Opn1sw	1.39	1.48	1/1.03
Per2	1.10	1.35	1.49
Rnr1#	1.23	1/1.08	1.69
Slc6a17	4.40*	11.3*	10.8*
Slc15a1	479.3*	472.2*	174.7*
Snf1lk	10.7*	11.8*	7.48*
St8sia5	1.64	2.27*	1.78
Tnf	1/1.03	1/ 4.68*	1/1.48
Tph1	1/1.05	1.07	1/1.21
Ttr	1/1.29	1.16	1/1.33
Ush2a	1/2.50*	1/1.53*	1/2.38*

Table 7. Functional grouping of genes that are night/day differentially expressed (N/D > 2 or < ½) or at high levels (rEx > 4) in the pineal gland. All genes appear in Tables 1, 3, S3 and S4.

Functional group	Gene Symbol
Specialized processes	
Immune response/ inflammation	*Abhd2*, And, Ahcy, Alms1*, ArHgef9, Bbs7, Bcar1, Btg2, C3, Ccl2, Ccl6, Ccl7, Ccl9, Ccrl2*, Cd1d1, Cd47, Cd74, Cd8a, Crcp, Ctsc, Ctss, Defb24, Dscr1, Fcer1a, Fras1*, Gdf15, Gem* Hivep1, Hivep2, Icsbp1, Ifi35, IfiTm1*, Ifnar1*, Igsf4a, Igsf9*, Igha, Il13ra2, Il17re, Il18, Il1rl1, Ilk, Impdh2, Inhbb, Irak2, Irf7, Ler3, Litaf, Lrrc8, Lta4h, Mal2, Mdk, Mina, Mmd2, Mox2, Mx2, Oit1*, Optn, Pcna, Plscr1, Pvr, Pvrl2, RT1-A1, RT1-A2, RT1-A3, RT1-Aw2, RT1-Bb, RT1-Da, Sct2, Sema3a, Serping1, Slfn3, Stch, Stip1, Tfp12, Tpm4, Usb2a, Vof16*
Melatonin synthesis	*Aanat, Acly, Asmt, Ddc, Gch1, Gchfr, Mat2a, Pcbd1, Tph1*
Photo-detection	Genes linked to photodetection in the pineal gland are considered to be highly expressed in both the pineal gland and retina; they are listed in Table 4 and Supplemental Data Table S5.
T3/RA Signaling	*Dio2, Hr, Rbp3, Rdh12*, Rorb*, Rxrg, Thrb, Ttr*
Non-specialized processes	
Adhesion	*Cdh22, Celsr32, Cml5, Cntn4, Dsc2, Eva*, Gja12*, Glycam1, Grn, Hnt, Mcam, Mfap4, Mpp4, Muc4, Nell2, Parvb, Pcdh21, Prph2, Pvr, Scarb2, Sdc4, Spon1, Ssx21p*
Cell cycle/ cell death	*Acom1, Acvr1, Aprin, Bag1*, Giklk, Casp7, Ccnd2, Cdc25a, Cdc5l, Cdk5, Cdkn1b, Cdkn1c, Cflar, Ches1*, Commd5, Csnk2a2, Ddit3, Dnm1, Dnm2, Elmo3, Faim, Gos2, Gadd45a, Gadd45b, Igf1r, Igfbpl1*, Jag1, Junb, Mad2l2, Mak10, Ntf3, Pafah1b1, Pard3, Pdia3, Plagl1, Ptgs2, Qscn6, Rarres1, Rgc32, Fhob, Slc31a1, Strn3, Tacc3, Vegfc*
Cytoskeleton	*Ap1g1, Baiapw, Bbs4*, Catna1, Clasp2, Clta, Col14a1*, Col3a1, Col4a3, Col8a1*, Cope*, Cpg2, Dnch1, Dncl2b*, Dncl2b*, Emilin1*, Emls, Fgd2*, Inb*, Flnc*, Fmod, Fni, Fscn2*, Hdac11*, Ka15, Kif1b, Kif22, Kif2c, Krt1-18, Krt1-19, Krt25, Lad1*, Lama2*, Lamb1-1*, Lap1b, Lcp1, Lix1, Lmod1, Lumk, Mapt, Marcks, Mfap5*, Mgp, Mrgl19, Mtap2, Mylip*, Nrap*, Pgea1, Rpl3, Sas, Selpl*, Sdo3, Spna2, Tctex1, Thbs4, Tmem16a*, Tmem22, Tpm4, Tuba4, Tubb5, Unc119, Vil2, Vim*
DNA modification	*Adprt, Blm*, Bnc2*, Cntn1, Commd1*,Ctps*, Herc3*, Hmgb2, Kpna2, Mcm4, Pcna, Prc1*, Prim1, Ptms, Rere, Thap4, Tlk1*, Top1, Tspyl4, Zdhhc22, Zfp143, Zfp162, Zfp238, Zfp36l1, Zhx1, Znf444, Zswim5**
Endothelium	*Esm1, Vegfb, Vegfc, Vwf*
Growth	*Efemt1, Egf, Egfr, Egfr1, Fgf1, Fgfr1, Gadd45g, Gdf15, Gfer, Grb2, Igf1r, Igfbp2, Igfbp6, Pdgfrl, Pgf, Tgfb1, Tgfbi, Vegfb, Vegfc*
Signaling	Calcium: *Atp2b3, Cabp1, Cacna1f, Cacna1g, Calm1, Camk1g, Camk2b, Cip98, Dcamkl1, Dcamkl3*
	Cyclic nucleotide: *Adcy8, Akap11, Cnga1, Cngb1, Creb3, Guca1a , Gucy1a3, Hcn1, Pde4b, Pde4d, Pde6b, Pde8b, Pde10a, Prkar2b, Prkca*
	G-protein: *Arf3, Arr3, Arl2bp, Arl6ip5, Gem, Gna12, Gnaq, Gnas, Gnat2, Gnaz, Gnb1, Gnb3, Gng11, Grk1, Pdc1, Rgs2, Rgs4, Rgs7, Rgs9, Rgs17, Sag1, Tyro3*
	Membrane receptors/ligands: *Acvr1, Adra1b, Adrb1, Agtrap, Bmp6, Chrna3, Chrnb1, Chrnb4, Crcp, Drd1a, Drd4, Ece1, Ednrb, Egf, Egfr, Fgf, Fgfr1, Fst, Fzd4, Grip2, Grm1, Grm2, Hcrtr1, Htr2c, Igf1r ,Igfbp2, Igfbp3, Igfbp5, Igfbp6, Lepr, Nog, Opn1sw, Prlr, Sort1,Vipr2*
	Lipid/ Phospholipid/cholesterol: *Abca1,Alox15, Cyp27a1, Ephx1, Inpp5e, Itpr1, Lta4h, Ltb4dh, Pa2g1b, Pik3r3, Pla2g5, Plcb1, Plcd4, Ptgds, Ptgis*
	MAP kinase: *Dusp1, Errfi1, Map3k5, Map3k6, Map4k1, Mapk14, Mapk6*
	Protein phosphorylation, serine/threonine: *Calm1, Camk1g, Camk2b, Cdk5, Cdkn1b, Crkas, Dcamkl1, Enh, Fez1, Gsk3b, Nell2, Pak2, Prkar2b, Prkca, Prkcdbp, Prkce, Prkcl1, Rock2, Sik2, Snrk, Stk2, Stk39*
	Protein phosphorylation, tyrosine: *Crkas, Efna5, Jak1, Kit, Ntrk2, Ntrk3, Ptp2E, Ptp4a1, Ptpn16, Ptprj, Ptprr, Ptp-Td14, Tyro3*
RNA modification	*Ankrd24*, Bfsp1, Bop1, Bzw2, Eif2ak4*, Eif2c2, Eif3s9, Eif4g2, Ell2, Hdac5, Polr2d*, Qtrt1, Rnase1, Rnase2*, Rpat1, Sfpq, Xpot**
Small molecule biology	Metal homeostasis, *Atp7b, Chordc1, Mt1a, Mt2, Slc30a1 Slc39a4*
	Ion homeostasis: *Atp1a1, Atp1b1, Atp1b2, Atp2a2, Atp2b1, Cacna1h, Cacnb2, Clcn3, Cnga1, Cngb1, Hcn1, Kcnab2, Kcne2, Kcnh6, Kcnj14, Kctd3, Scn7a, Slc12a2, Slc12a5, Slc17a6, Slc24a1*
	Solute transport: *Slc2a1, Slc2a4, Slc3a1, Slc4a2, Slc4a4, Slc6a6, Slc7a1, Slc7a7, Slc12a2, Slc12a5, Slc14a1, Slc15a1, Slc16a1, Slc16a6, Slc21a1, Slc21a7, Slc22a1, Slc25a10, Slc29a1, Slc30a1, Slc34a1*
Transcription Factors	*Arntl, Bhlhb3, Cebpb, Crem, Cbx5, Cry2, Crx, Datf1, Eya2, Fosl2, Foxd1, Hdac5, Homer1, Homer2, Hr, Isl2, Jun, Junb, Mitf, Msx1, Neurod1, Nr1d2, Nr1h4, Nr2f6, Nr4a1, Nr4a3, Otx2, Pax4, Pax6, Per2, Ptch1, Rax, Rorb, Rxrg, Thrb,*
Vesicle biology	*Cadps, Chga, Chgb, Clta, Cltb, Dnm1, Dnm2, Dnm3, Lphn2, Ptprn, Scg2, Scg3, Snap23, Snap25, Sny2, Stx3, Sv2b, Syt4*

Figure 1

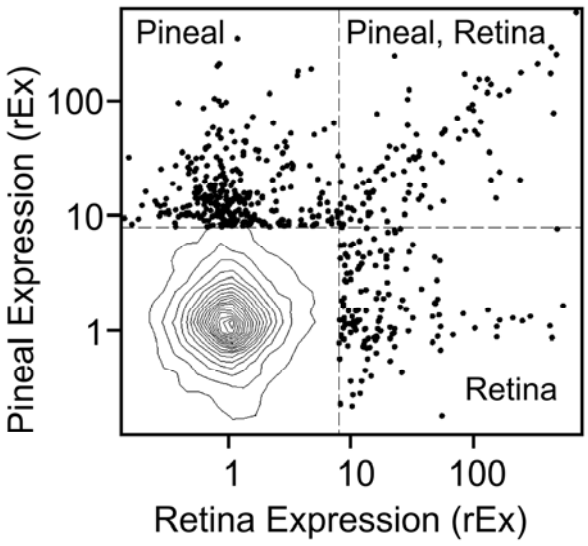

Figure 2 (Panel A)

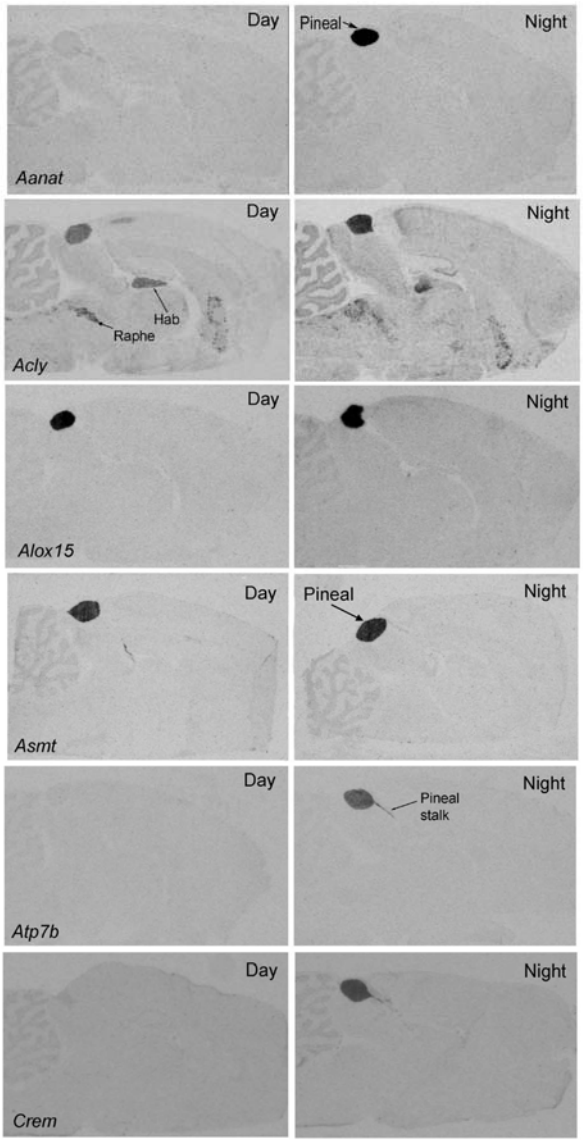

Figure 2 (Panel B)

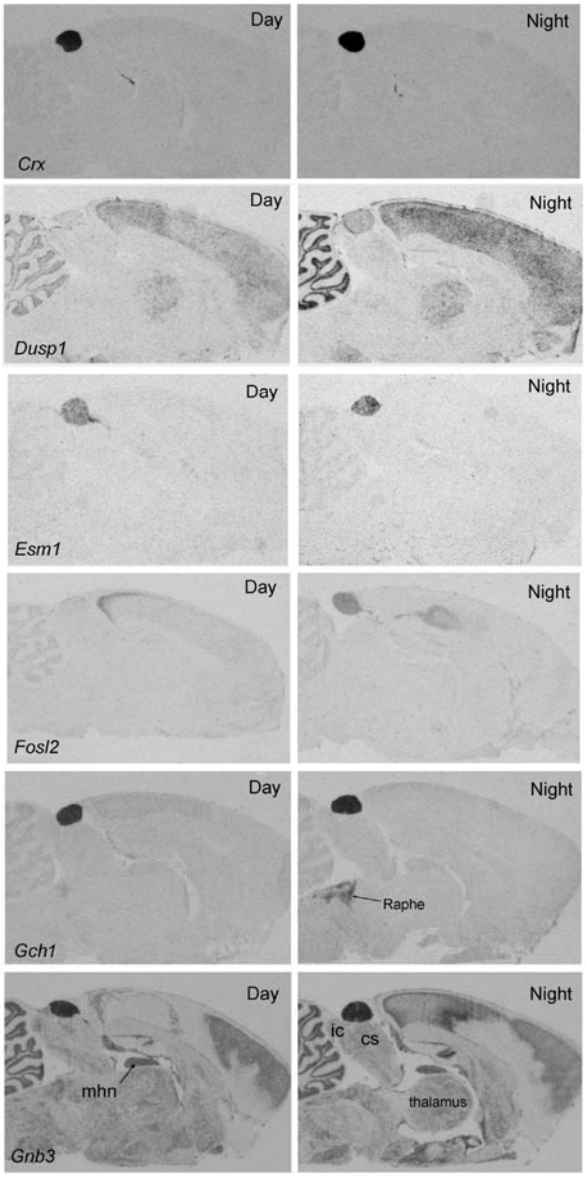

Figure 2 (Panel C)

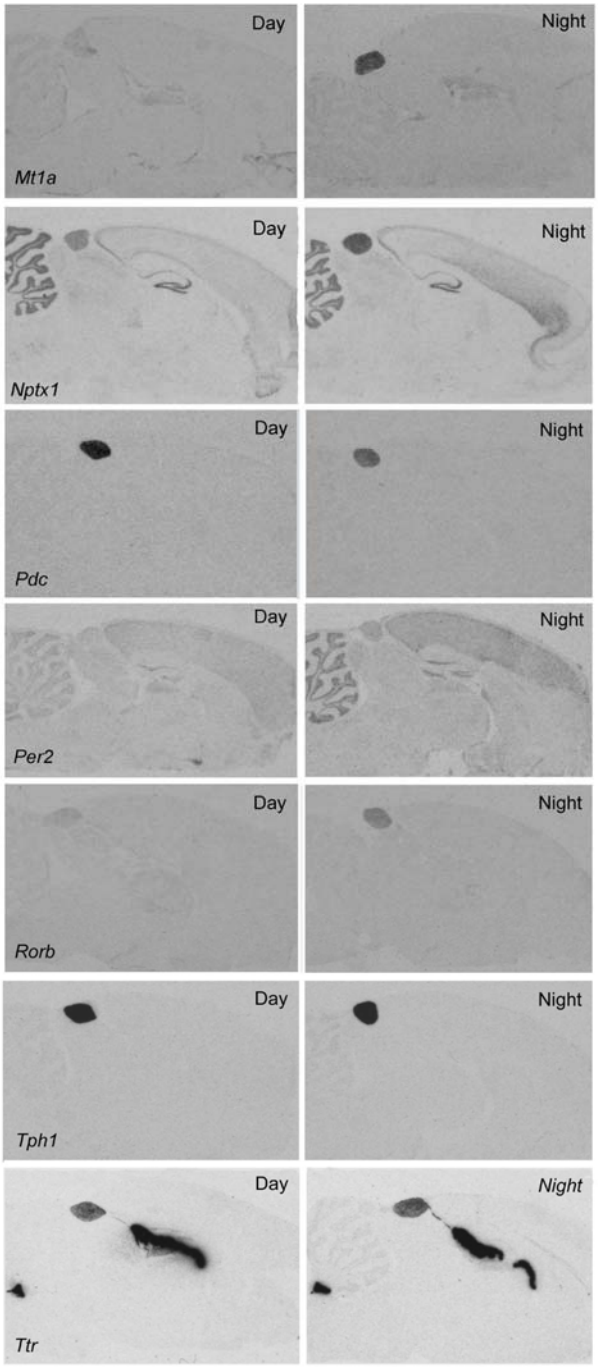

Figure 3

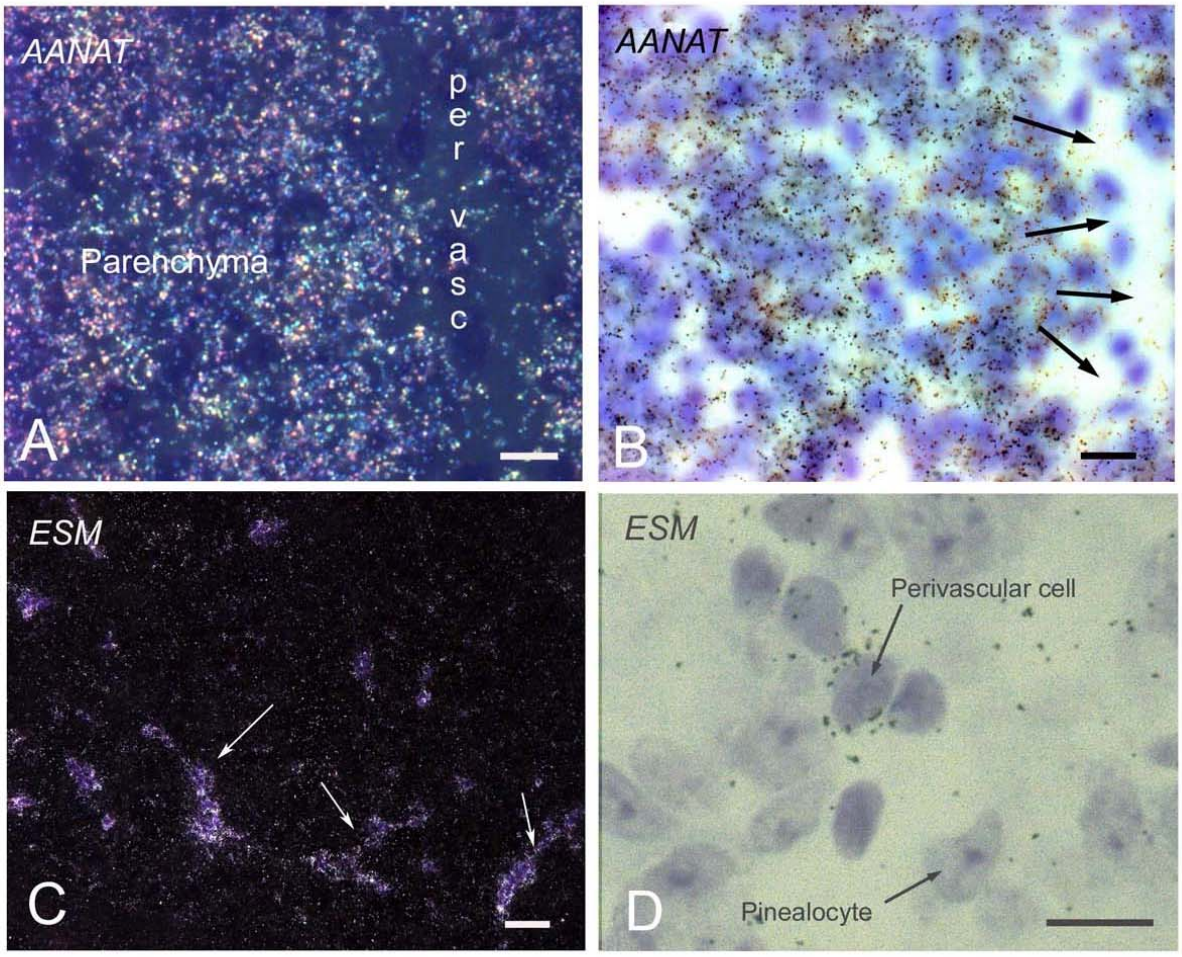

Figure 4